IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF DISTINCT HIGH MOLECULAR WEIGHT FORMS OF NEUROPHYSIN AND SOMATOSTATIN IN MOUSE HYPOTHALAMUS EXTRACTS

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Received 14 November 1978

1. Introduction

The biosynthesis of numerous secretory proteins and peptide hormones appears to proceed via higher molecular weight precursors [1–4]. These larger forms, elaborated on the rough-surfaced endoplasmic reticulum [5,6], undergo several transformations, named processing, which generally lead to the unmasking of biologically active 'final' compounds. Biosynthetic evidence [7,8] for such a general scheme, derived from in vivo pulse chase experiments, seems to apply to the biogenesis of the neurophysins, neurohypophyseal hormone-associated proteins, synthesized by neurosecretory neurons of the magnocellular system in the supraopticus and paraventricular nuclei of the hypothalamus. In the course of our biochemical approach to the characterization of such putative pro-neurophysins we were able to detect, using a radioimmunological assay, neurophysin-like materials with molecular weight '17 k' and $\geqq$ '20 k'. The possible plurifunctionality of the immunologically related high molecular forms of neurophysin isolated from mouse hypothalamus extracts was tested. Besides this putative '17 k' pro-neurophysin, high molecular weight forms of materials immunologically related to somatostatin (somatotrope releasing inhibitor factor: SRIF) could be found. We show evidence that the neurophysin-like and SRIF-like immunoreactivity are associated with distinct molecules and that the putative '17 k' pro-neurophysin probably contains carbohydrate moieties.

2. Materials and methods

Phenylmethyl-sulfonylfluoride (PMSF), Trasylol, insoluble concanavalin A (Con A)–agarose, and α-methyl-D-mannoside were purchased from Sigma (St Louis). Triton X-100 was supplied by Calbiochem (San Diego). The antiserum A5 IV directed against neurophysin was a gift of Dr Legros (Liege) [9]. The antisomatostatin 3638 antiserum was provided by the URIA (Dr Dray) Institut Pasteur, Paris.

All experiments were performed at 4°C. Mice hypothalami (50 fragments) were removed under a Nikon type 102 binocular immediately after sacrifice and homogenized with a Potter Elvejhem in 5 ml 0.1 N HCl, 8 M urea, 5 mM PMSF, 130 units/ml Trasylol. The extract was frozen in liquid nitrogen and thawed (3 times), then centrifuged 1 h at 100 000 × *g* in a SW 50 rotor (Beckman L5 50). The supernatant was filtered on a Bio-gel P-10 or a Sephadex G-50 column equilibrated and eluted with 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF and 130 units/ml Trasylol. All fractions were tested by a radioimmunoassay procedure (RIA) using antisera directed against neurophysin (A5 IV) or somatostatin (3638). The free iodinated tracer was separated from the antibody-bound tracer by means of zyrconyl phosphate for neurophysin [10] and propanol precipitation for somatostatin. The insoluble concanavalin A column (Con A–agarose complex) was prepared as in [11]. The samples were dissolved in the Con A buffer (0.01 M Tris–HCl, 0.7 mM

$MgCl_2$, 1.0 mg/ml bovine serum albumin, 1.0 M NaCl, 0.1% Triton X-100, pH 7.4) and layered on the 1 ml columns. After washing with 30 ml Con A buffer, the specifically bound material was eluted with 0.2 M α-methyl-D-mannoside in Con A buffer, followed by 1 M α-methyl-D-mannoside in the same buffer. After this treatment, any material still bound to the column was eluted with 0.1 M acetic acid. All fractions were tested by the RIA procedure.

3. Results

The elution profile in fig.1A indicates clearly that besides the major immunoreactive peak corresponding to the 10 k mol. wt species (neurophysin) two other peaks (≧ '20 k' and ≃ '17 k') can be detected. The relative amount of the ≧ '20 k' and ≃ '17 k' species was dependent largely upon the extraction procedure. When membrane centrifugation in isotonic sucrose buffer was performed before the 100 000 × *g* centrifugation, the ≧ '20 k' species appeared more intensely suggesting a membrane-mediated degradation of this high molecular weight form. In addition, the proportion of '17 k' species increased significantly consecutive to protease inhibitor addition. The molecular weight homogeneity of this compound was demonstrated by further Bio-gel P-10 filtration after 16 h exposure to 8 M urea [12] (fig.1B). This demonstrates that these forms of neurophysin-like material are not neurophysin aggregates or non-covalent polypeptide–neurophysin complexes. An analysis of the same hypothalamic extract for SRIF-like immunoreactivity showed a pattern suggesting multiple high molecular weight forms with a major component distinct from the '17 k' neurophysin-like peak (not shown). In order to obtain an improved separation of these components the same analysis was conducted on a Sephadex G-50 column.

Figure 2A shows a pattern indicating three major immunoreactive components of molecular weight respectively evaluated to '15 k', '8 k' and a compound co-eluting with somatostatin. The molecular weight homogeneity of these '15 k' and '8 k' species (fig.2B,2C) was established by the same procedure used for the '17 k' putative proneurophysin after 48 h exposure to urea. As can be observed on both

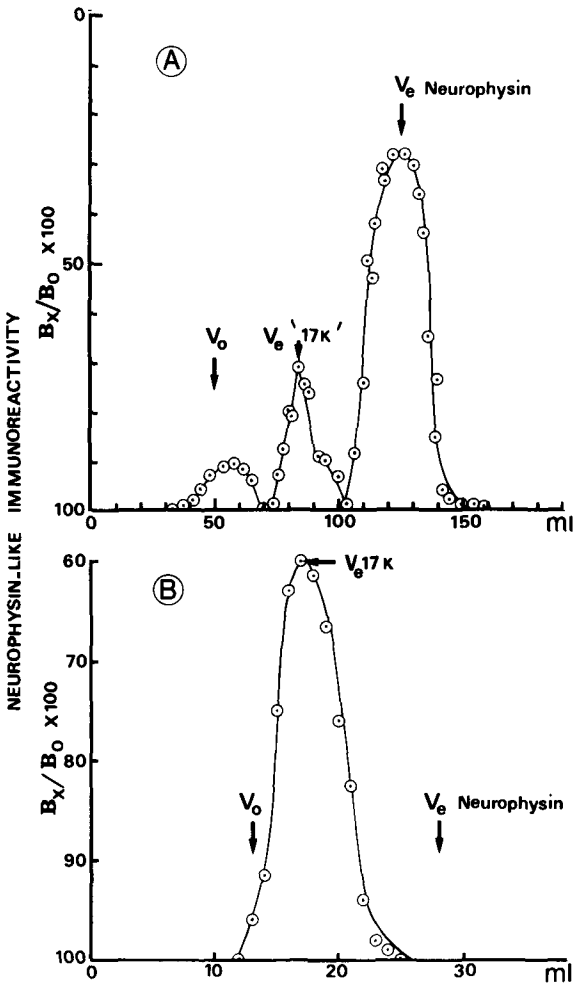

Fig.1. Immunological evidence for high molecular weight forms of neurophysin. (A) Gel filtration fractionation (Bio-gel P-10, 40 × 2.6 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF and 130 units/ml Trasylol) of the neurophysin-like immunoreactive material (as determined by RIA on each 1 ml eluted fraction) after extraction in 0.1 N HCl containing 8 M urea, 5 mM PMSF, 130 units/ml Trasylol, of mouse hypothalami. The discrete immunoreactive peak eluted at the V_0 corresponds to a material ≥ 20 k. (B) Gel filtration (Bio-gel P-10, 19 × 1.6 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF, 130 units/ml Trasylol) of '17 k' species after a 16 h treatment in 8 M urea. Immunoreactive material was found only at the V_e of the '17 k' species. The B_x/B_o × 100 ratio represents the percentage of the antibody-bound [^{25}I]neurophysin tracer.

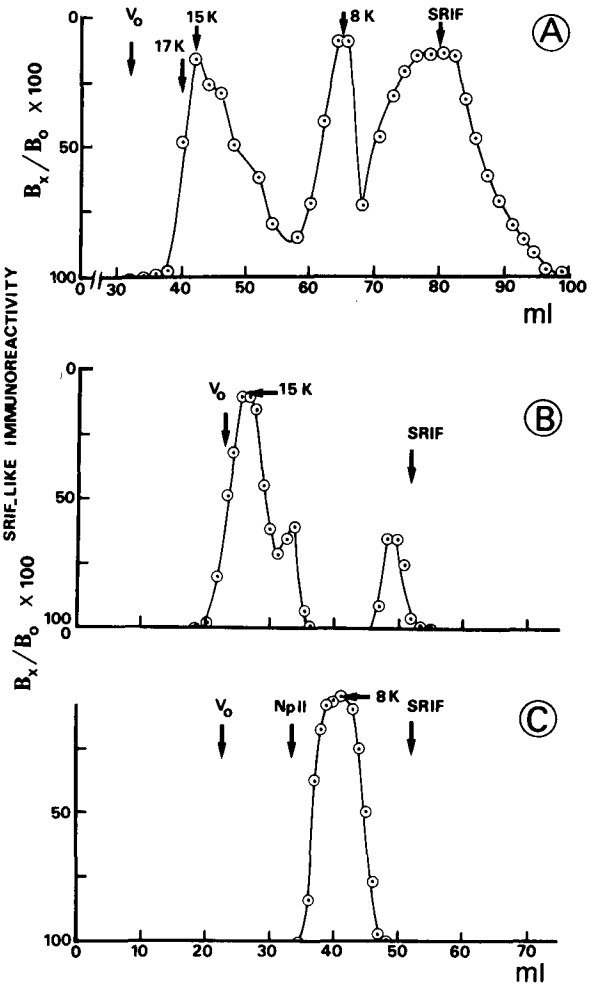

Fig.2. Immunological evidence for high molecular weight forms of somatostatin. (A) Gel filtration fractionation (Sephadex G-50, 75 X 1.2 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF, 130 units/ml Trasylol) of the somatostatin-like material (as determined by RIA on each 1 ml eluted fraction) after extraction in 0.1 N HCl containing 8 M urea, 5 mM PMSF, 130 units/ml Trasylol, of mouse hypothalami. (B) Gel filtration (Sephadex G-50, 45 X 1.2 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF, 130 units/ml Trasylol) of the '15 k' species after a 48 h treatment in 8 M urea. The recovery of the total somatostatin immunoreactivity was > 90%. (C) Gel filtration (Sephadex G-50, 45 X 1.2 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF, 130 units/ml Trasylol) of the '8 k' species after a 48 h treatment in 8 M urea. The recovery of the immunoreactive somatostatin-like material was ⩾ 90%. The B_x/B_0 X 100 ratio represents the percentage of the antibody-bound [^{125}I]somatostatin tracer.

fig.2A,2B the '15 k' peak reveals a shoulder (≃ 10 k) possibly corresponding to an intermediary form.

The immunological identity between the '17 k' neurophysin-like material and neurophysin on the one hand and of these '15 k' and '8 k' somatostatin-like compounds with somatostatin on the other was established by direct RIA competition assay. Displacement curves parallel to the standard curve were obtained in both cases (fig.3A,3B). After incubation

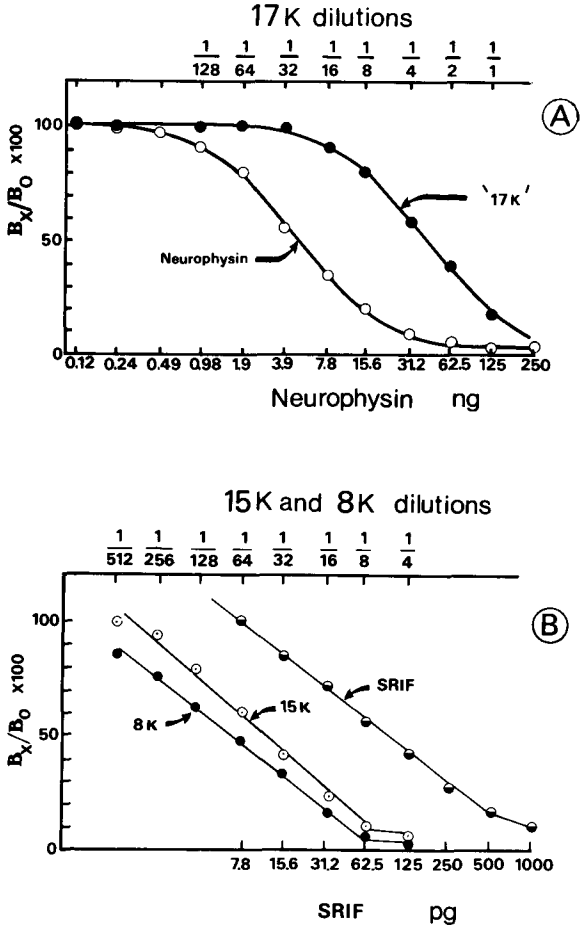

Fig.3. Immunological characterization of the '17 k' neurophysin-like and '15 k' and '8 k' somatostatin-like materials. (A) Comparison of the cross reactivity of mouse neurophysin (○) and '17 k' species (●) as studied by RIA using antiserum (A5 IV serum obtained from Dr Legros) exhibiting no species specificity. (B) Comparison of the cross-reactivity of reference somatostatin (⊖) the '15 k' (⊙) and the '8 k' (●) species as studied by RIA using a somatostatin antiserum 3638 and Tyr-*N*-somatostatin as tracer.

of the putative '17 k' pro-neurophysin and '15 k' somatostatin-like material with trypsin (trypsin dilutions 1/100 or 1/1000; incubation at 37°C; 10 min, 30 min, 60 min) only small molecular weight species, corresponding to degradation forms of neurophysin and somatostatin could be found, possibly suggesting that conversion of these high molecular weight forms of neurophysin-like and somatostatin-like material in final components is not mediated by a trypsin-like cleavage.

Glycosylation of biosynthetic precursors forms of secretory peptides has been shown [11,13,14] to be involved at various stages of the secretion process. Affinity chromatography on Con A–agarose might provide a simple mean of determining whether these high molecular weight forms of neurophysin or somatostatin were glycoproteins. The '15 k' SRIF like compound purified by Bio-gel P-10 column (fig.2B) were lyophylized, diluted in Con A buffer and analyzed on a column of Con A–agarose as in section 2. As shown in the fig.4A the '15 k' product was not bound to the affinity adsorbant and almost entirely eluted from the column by the washing buffer. In contrast a major part of the starting 'neurophysin-like' material recovered as a '17 k' peak was found to be eluted from the affinity adsorbant only by 0.2 M α-methyl-D-mannoside, fig.4B, suggesting carbohydrate mediated interactions with the adsorbant.

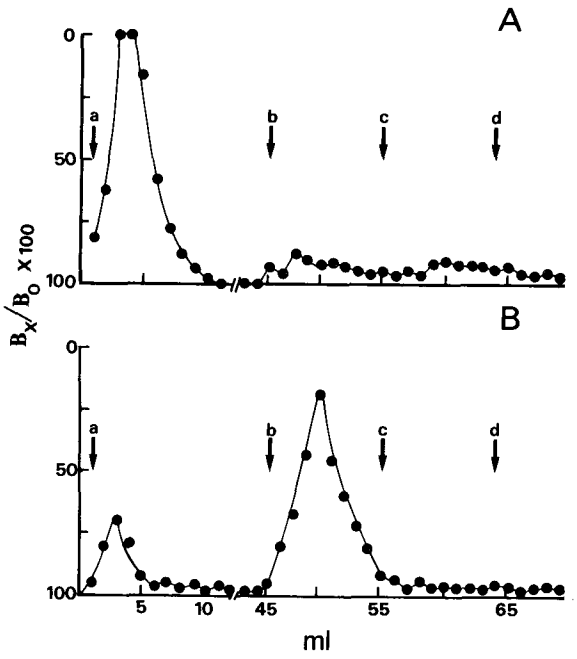

Fig.4. Affinity chromatography on a Con A–agarose adsorbant. (A) The lyophylized '15 k' somatostatin-like material dissolved in 1 ml of the Con A buffer (0.01 M Tris–HCl, 1.0 M NaCl, 0.7 mM $MgCl_2$, 0.1% Triton X-100, 1 mg/ml SAB, pH 7.4) was adsorbed on a Con A–agarose column (1 ml total vol.) equilibrated in the same buffer. The column was washed with 30 ml Con A buffer (a) and eluted with 10 ml 0.2 M α-methyl-D-mannoside in Con A buffer (b), followed by 10 ml 1 M α-methyl-D-mannoside in Con A buffer (c). The column was then washed by 10 ml 0.1 M acetic acid (d). Immunoreactive material was determined by RIA on each 1 ml fraction. The recovery of immunoreactive somatostatin-like material was ⩾ 95%. (B) The lyophylized '17 k' neurophysin-like material dissolved in 1 ml Con A buffer was adsorbed and eluted from the column as described in fig.4A. The recovery of immunoreactive neurophysin-like material eluted by 0.2 M α-methyl-D-mannoside buffer was ⩾ 90%.

4. Discussion

The above-reported observations reinforce and extend the previous findings from pulse-chase analysis by others [7,8,15] that neurophysin biosynthesis may occur via higher molecular weight precursors. In addition to the '17 k' form, we are able also to detect a ≧ '20 k' species which seems to be relatively labile and could preferably be isolated as a product of in vitro translation of reticulum-bound polysomes from hypothalamic neurons. Iso-electricfocusing of the '17 k' species reveals some heterogeneity on the basis of the net charge and suggests that two distinct high molecular weight forms of neurophysin are present [16,17] in keeping with the idea supporting that the two major neurophysins found in the mouse hypothalamo-neurohypophysis tract are synthesized via two pro-neurophysins. Our observations also bring an additional element suggesting that the high molecular form of neurophysin is glycosylated. This suggests that the estimated molecular weight ('17 k') of this form is, possibly, not exact and remains to be determined by methods which are not impaired by the presence of carbohydrate moieties on the molecule [11]. The question of possible common precursor molecules for the vasopressin–neurophysin and/or ocytocin–neurophysin pairs still remains open.

In situ observations of simultaneous positive reactions of supraoptic and paraventricular nuclei of rat hypothalamus with anti-SRIF and anti-neurophysin antibodies [18,19] might be taken as suggestive of common loci of synthesis, and eventually by reference to other plurifunctional pro-hormones [20,21], of a common precursor molecule. Our analysis of the extract with anti-SRIF anti-serum delineates the presence of multiple high molecular weight forms. But two sets of evidence suggest that SRIF-like and neurophysin-like species belong in two distinct families of molecules. These include:

(i) The different elution profile;
(ii) The absence of any detectable affinity of the '15 k' SRIF-like form on the Con A adsorbant in contrast with the behaviour of the '17 k' neurophysin-like material.

Of interest are the very recent observations made on the canine pancreas [22] describing high molecular weight forms (12 k and 3.5 k) of SRIF comparable with ours. Some apparent differences might be due to posttranslational modifications of products, resulting from different extraction procedures.

More detailed biochemical analysis of those pro-hormones molecules, in progress in our laboratory, will permit to establish more definitive structural relationships between the putative precursors and secretory products.

Acknowledgements

This work was supported in part by the Université P. et M. Curie, the Centre National de la Recherche Scientifique (RCP 220 and ERA 693), the Délégation Générale à la Recherche Scientifique et Technique (contract 76–7–1188), the Departement de Biologie du Commissariat à l'Energie Atomique de Saclay (by a grant for radiochemicals and a doctoral fellowship to M.L.) and the Fondation pour la Recherche Médicale Française. We wish to thank Dr Pierre Fromageot for his continuous support and Professor R. Guillemin for stimulating suggestions. The help of Drs Gros and Pradelles with the somatostatin RIA is kindly acknowledged.

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